Expression of vascular endothelial growth factor protein in mRNA-transfected human periodontal ligament cells

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

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A Thesis Submitted in Partial Fulfillment of the Requirements
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การแสดงออกของวาสคูลาร์เอนโดทีเลียลโกรทแฟกเตอร์โปรตีนจากการนำเอ็มอาร์เอ็นเอเข้าสู่เซลล์เอ็นยึดปริทันต์ของมนุษย์

น.ส.มานา นราธิปกร

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มานา นราธิปกร : การแสดงออกของวาสคูลาร์เอนโดทีเลียลโกรทแฟกเตอร์โปรตีนจากการนำเอ็มอาร์เอ็นเอเข้าสู่เซลล์เอ็นยึดปริทันต์ของมนุษย์. (Expression of vascular endothelial growth factor protein in mRNA-transfected human periodontal ligament cells) อ.ที่ปรึกษาหลัก : ศ. ทพญ. ดร.รังสินี มหานนท์

ในปัจจุบันการรักษาเพื่อฟื้นฟูเนื้อเยื่อปริทันต์ที่ถูกทำลายจากโรคปริทันต์อักเสบให้กลับมาอย่างสมบูรณ์ยังคงเป็นวิธีที่เป็นไปได้ยาก เนื่องจากวิธีการที่ใช้อยู่ให้ผลการรักษาที่ไม่แน่นอนและไม่สามารถคาดเดาได้ การใช้เอ็มอาร์เอ็นเอ (mRNA) อาจจะเป็นนวัตกรรมใหม่ที่เหมาะสมในการฟื้นฟูเนื้อเยื่อ การศึกษานี้มีวัตถุประสงค์เพื่อประเมินความสามารถของเซลล์เอ็นยึดปริทันต์ในการผลิตโปรตีนวาสคูลาร์เอนโดทีเลียลโกรทแฟกเตอร์ (vascular endothelial growth factor, VEGF, วีอีจีเอฟ) ภายหลังจากการนำส่งเอ็มอาร์เอ็นเอที่ถูกดัดแปลงเบสเป็นซูโดยูริดีน (pseudouridine) ซึ่งเข้ารหัสด้วยวีอีจีเอฟเข้าสู่เซลล์เอ็นยึดปริทันต์ของมนุษย์ และนำโปรตีนวีอีจีเอฟที่ถูกผลิตขึ้นมาทดสอบประสิทธิภาพในการสร้างเส้นเลือดใหม่ในเนื้อเยื่อโคริโออัลแลนโทอิคของเอ็มบริโอไก่หรือแคมเอสเส (chick chorioallantoic membrane or CAM assay) VEGF-mRNA ที่ห่อหุ้มด้วยไลโปแฟคตามีน 2000 (VEGF mRNA-L2000) และ L2000 จะถูกนำส่งเข้าไปยังเซลล์เอ็นยึดปริทันต์ที่ได้มาจากเนื้อเยื่อปริทันต์ของผู้ป่วยที่มีสภาวะปริทันต์ปกติ ภายหลัง 24 ชั่วโมงทำการเก็บเซลล์ (cells) และส่วนใส (supernatant) จากการเพาะเลี้ยงเซลล์เพื่อนำไปวัดปริมาณของโปรตีน VEGF ด้วยวิธีอีไลซา (ELISA) และทำการทดสอบการมีชีวิตของเซลล์ด้วยอะลามาร์บูล (Alamar Blue assay) หลังจากนั้นนำส่วนใสที่ได้จากกลุ่มเอ็มอาร์เอ็นเอ กลุ่มควบคุม L2000 และกลุ่มควบคุมดีพีบีเอส (DPBS) ใส่ลงบนกระดาษกรองเพื่อมาทดสอบใน CAM assay ในเอ็มบริโออายุ 8 วัน และติดตามผลนาน 3 วัน เพื่อประเมินการสร้างเส้นเลือดที่เพิ่มขึ้นจากวันที่ 8 ถึง 11 จากภาพถ่ายจากกล้องจุลทรรศน์แบบสเตอริโอ ผลการศึกษาพบว่าเซลล์เอ็นยึดปริทันต์ที่ถูกนำส่งด้วย VEGF mRNA จะเข้าสู่เซลล์ และสามารถผลิตโปรตีน VEGF ได้มากกว่ากลุ่มควบคุมทั้ง 2 กลุ่ม (L2000 และ DPBS) อย่างมีนัยสำคัญ ($p < 0.001$) โดยการใช้เอ็มอาร์เอ็นเอไม่ส่งผลต่อชีวิตของเซลล์ และเมื่อนำโปรตีนที่ได้มาทดสอบใน CAM assay พบว่ากลุ่ม VEGF mRNA มีการสร้างเส้นเลือดเพิ่มขึ้นได้มากกว่ากลุ่มอื่นอย่างมีนัยสำคัญ ($p < 0.001$) สรุปได้ว่าการนำส่งเซลล์เอ็นยึดปริทันต์ด้วย VEGF mRNA สามารถผลิตโปรตีน VEGF mRNA ได้ในปริมาณที่สูง และสามารถส่งเสริมให้เกิดการสร้างเส้นเลือดเพิ่มขึ้นได้ในเนื้อเยื่อโคริโออัลแลนโทอิค ซึ่งมีความเป็นไปได้ที่จะนำ mRNA เทคโนโลยีแพลทฟอร์มมาใช้ในการรักษาฟื้นฟูเนื้อเยื่อปริทันต์ที่ถูกทำลายจากโรคปริทันต์อักเสบได้

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สาขาวิชา	ปริทันตศาสตร์	ลายมือชื่อนิสิต ....
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Mana Naratippakorn : Expression of vascular endothelial growth factor protein in mRNA-transfected human periodontal ligament cells. Advisor: Prof. RANGSINI MAHANONDA, D.D.S., M.Sc., Ph.D.

The complete regeneration of periodontal tissues following current periodontal therapy remain challenging and unpredictable. Nucleoside-modified messenger RNA (mRNA) technology can be a promising novel platform in regenerative medicine. The aims of this study were to evaluate whether pseudouridine modified mRNA encoding vascular endothelial growth factor (VEGF) could induce VEGF production in human periodontal ligament cells (PDLCs) and this translated protein function by promoting *in vivo* blood vessel formation using chorioallantoic membrane (CAM) assay. Isolated PDLCs from healthy periodontal tissue were transfected with modified mRNA encoding VEGF (VEGF mRNA) complexed with a transfecting agent, Lipofectamine 2000 (L2000) and L2000 alone (control). Supernatants collected at 24 hours (h) after transfection were evaluated for protein production by ELISA and cell viability by Alamar Blue assay. The supernatants of the VEGF mRNA-L2000, L2000 (L2000 control), and DPBS (negative control) were applied on filter papers, individually placed these grafts on to the CAM surface through the window on day 8 of embryonic development (E8) and incubated for another three days. Angiogenesis assessment, counting number of blood vessels convergence to the grafts, was carried out by photographed with stereomicroscopic on E8 and E11. The result showed that PDLCs, transfected with mRNA encoding VEGF, produced high level of VEGF protein than controls at 24 h ($p < 0.001$). The transfection of mRNA encoding VEGF showed negligible effect on PDLC viability. When supernatants were applied in CAM assay, translated protein VEGF protein was able to significantly induce blood vessel formation ($p < 0.001$). In conclusion, modified mRNA encoding VEGF promoted VEGF production and had angiogenic properties, increased blood vessel formation in the CAM. Thus, this mRNA platform technology may allow future application as a novel therapeutic platform for periodontal regeneration.

Field of Study:	Periodontics	Student's Signature ....
Academic Year:	2021	Advisor's Signature ....

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CHAPTER I

INTRODUCTION

Background and Rationale

In 2017, over 800 million global population (approximately 11%) were diagnosed with severe periodontitis (GBD Oral Disorders Collaborators, 2020). Periodontitis is a chronic inflammatory disease which results from host immune response to dental plaque. The disease destroys periodontium, a tooth supporting structure including gingiva, periodontal ligaments, cementum and alveolar bone. In severe periodontitis, such damaging inflammation could lead to tooth loss.

Initial therapy for periodontal treatment consists of removal of dental plaque and calculus by scaling and root planing, allowing resolution of inflammation to occur. In the advanced form of periodontitis, common surgical procedures utilized for periodontal regeneration include guided tissue regeneration and bone grafting (Bowers et al., 1982; Nyman et al., 1982). However, these treatments provide unpredictable with mixed clinical outcomes and costly (Avila-Ortiz et al., 2015; Kao et al., 2015). Therefore, novel periodontal treatments need to be developed to restore the damaged or lost tissues to their original form and function.

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Current therapeutic strategies for periodontal regeneration are based on the key concept of tissue engineering using stem cells, scaffolds, and growth factors in the context of an adequate blood supply (Larsson et al., 2016). Growth factors are important tools to stimulate multipotent cells in periodontal tissues to proliferate and differentiate into the desired soft and hard tissues. Currently, only a few recombinant human growth factor proteins including platelet derived-growth factor-BB (GEM-21®), bone morphogenetic protein-2 (INFUSE®), and fibroblast growth factor-2 (Regroth®) are used as

an adjunct to periodontal surgery in periodontal defects and dental implant ridge augmentation. In spite of their attractive properties of these protein growth factors, several studies showed inconclusive clinical efficacy of recombinant growth factor application in periodontal regeneration (Donos et al., 2019). Half-life of growth factors *in vivo* is relatively short usually ranging from several hours to days (Rennel et al., 2008), therefore, supraphysiologic dose or several administrations are required (Zara et al., 2011). Such high dose of growth factors may cause undesirable side effects and increase the cost of therapy.

Gene therapy approach may provide better bioavailability of growth factor within the damaged tissue such as periodontal defects in periodontitis patients. This approach involves the delivery of nucleic acids, either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) encoding growth factors into patient's cells and the cells become the growth factor protein factory. mRNA-based technology has advantage over DNA-based technology since mRNA do not enter the nucleus (no risk of mutagenesis) and require simple and uncomplicated methods (Warren & Lin, 2019; Youn & Chung, 2015). Of note, nucleoside-modified mRNA platform has proven to be a successful vaccine modality against COVID-19, demonstrating safety and high efficacy in humans (Baden et al., 2021; Polack et al., 2020). It is the first mRNA product approved by FDA (U.S. Food and Drug Administration). The same fundamental technology platform could be applied to facilitate the development of mRNA-based regenerative therapy.

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Pioneer group using mRNA encoding vascular endothelial growth factor (VEGF mRNA) demonstrated promising data of heart tissue regeneration in rat and swine myocardial infarction (Carlsson et al., 2018; Zangi et al., 2013). VEGF is an important angiogenic growth factor which induce new blood vessel formation, thus providing oxygen and nutrients to damaged tissue and facilitating

wound healing and regeneration. At present, this VEGF mRNA (AZD8601, Moderna and AstraZeneca) is being tested in human clinical Phase 2a trial (AstraZeneca, 2021), thus suggesting the therapeutic potential of VEGF-A mRNA for regenerative angiogenesis.

Our research team aims to develop nucleoside-modified mRNA encoding growth factors such as VEGF for periodontal regeneration. In this present study, we investigated protein expression after VEGF mRNA transfection in target cells, human periodontal ligament cells (PDLCs) and analyzed biological activity, angiogenic effect of the translated protein using chorioallantoic membrane (CAM) assay.

Objective

1) To evaluate VEGF protein production from culture supernatant of nucleoside-modified mRNA encoding VEGF-transfected PDLCs.
2) To evaluate angiogenic effect of translated protein using CAM assay.

Hypothesis

Administration of nucleoside-modified mRNA encoding VEGF can transfected PDLCs and express VEGF protein *in vitro*. This culture supernatant of translated protein can enhance blood vessel formation in CAM assay.

Field of research

In vitro and *in vivo* study of nucleoside-modified mRNA encoding VEGF formulated with Lipofectamine™ 2000.

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Inclusion criteria

Human periodontal ligament cells were obtained from healthy periodontal patients undergoing tooth extraction due to orthodontic reasons or wisdom teeth.

Limitation of research

This study is an *in vivo* pilot study with a small sample size, thus increasing a sample size number should be employed in the future study.

Application and expectation of research

This research will provide information regarding the potential use of VEGF mRNA for periodontal regeneration.

Keywords

mRNA, vascular endothelial growth factor, angiogenesis, CAM assay, periodontal regeneration

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CHAPTER II

LITERATURE REVIEW

Periodontitis

Periodontitis have historically been considered the most important oral health burden. Although, medical advances improve efficiency of oral treatment, in 2017, over 800 million people worldwide are still diagnosed severe periodontitis. The prevalence of severe periodontitis increases gradually with age, reached its peak at age 60 to 64 years (GBD Oral Disorders Collaborators, 2020). In Thailand, 18% of the population are recognized severe periodontitis (Oral health survey of Thailand, 2017).

Periodontium is the attachment apparatus, including gingiva, periodontal ligaments, cementum and alveolar bone, which hold teeth firmly to jawbone and provide a barrier from the oral microflora. Naturally, dental plaque constantly accumulate on the tooth surface in close proximity to gingiva and host response to microbial plaque could lead to gingival inflammation (gingivitis). If left unremoved (tooth brushing and flossing) or untreated (scaling and root planing), inflammation in the superficial tissue-gingiva may progress and cause damaging inflammation to deeper tissues-periodontal ligaments, cementum and bone. And this advanced periodontal disease is called periodontitis (Fig. 1). Current hypothesis of immunopathogenesis of periodontitis involves chronic inflammatory immune response to dysbiotic microbial plaque. Keystone pathogens, such as *Porphyromonas gingivalis*, play a crucial role in dysbiotic plaque. They could orchestrate commensal bacteria to become pathobionts, disrupt homeostasis, and impair host immune response leading to periodontal destruction. In severe periodontitis, such damaging inflammation could lead to tooth loss (Hajishengallis, 2014).

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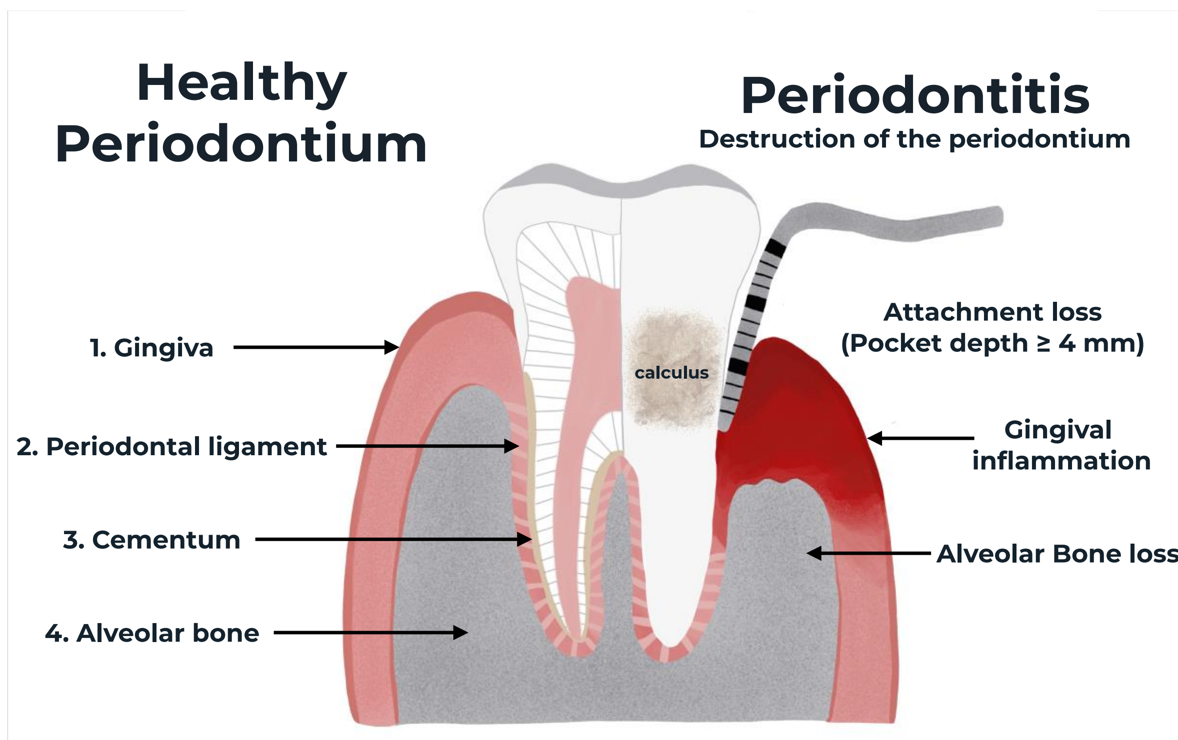

Figure 1. Healthy periodontium vs periodontitis. Periodontium consists of gingiva, periodontal ligament, cementum and alveolar bone. Periodontitis, a severe form of periodontal disease, with plaque and calculus deposits, manifests gingival inflammation, loss of clinical attachment with greater 4 mm probing depth and bone loss.

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Treatment of Periodontitis

Initial therapy for periodontitis consists of scaling and root planing and oral hygiene instruction. The primary purpose of periodontal therapy is to resolve inflammation in order to arrest disease progression and prevent its recurrence. However, the ultimate goal of periodontal treatment is to regenerate destructive periodontium, which helps the periodontium function properly and improve the prognosis of the teeth.

Common clinical procedures for periodontal tissue regeneration include guided tissue regeneration and bone grafting. Guided tissue regeneration can be performed with the use of barrier

membrane which act as epithelium exclusion from the periodontal defects and help maintaining the space for periodontal regeneration (Nyman et al., 1982). Bone grafting serves as a scaffold for bone regeneration, allowing osteoprogenitor cell migration and ingrowth within the graft, and preventing collapse of the flap (Mellonig, 1984). Although most patients respond favorably to the treatment, some do not. This is because there are multiple factors related to patients (e.g. smoking, diabetes mellitus and compliance), defect sites (e.g. bony morphology, root topography and gingival biotype) and surgical techniques (Reynolds et al., 2015). As a result, it is still challenging to achieve complete or even partial regeneration (Avila-Ortiz et al., 2015; Sculean et al., 2015). Moreover, this technique requires a long time to heal and high cost.

Growth factors in tissue engineering

The current therapeutic strategies for periodontal tissue regeneration have been based on the key concept of "tissue engineering" regarding the use of stem cells, scaffolds, and growth factors in the context of blood supply (Vacanti & Langer, 1999) (Fig. 2). As for the growth factors, in spite of their attractive properties as a tool to stimulate multipotent cells within periodontal tissues to proliferate and differentiate into desired soft and hard tissues, their half-life of growth factors *in vivo* is relatively short, usually ranging from minutes to hours (Rennel et al., 2008). Consequently, supra-physiologic dose or multiple administrations are required (Zara et al., 2011). Such high dose of growth factors may cause undesirable side effects and increase the therapy costs (Carragee et al., 2011). Inconclusive clinical efficacy of growth factors in periodontal and bone regeneration has been reported in various studies. So far, only recombinant human platelet derived-growth factor-BB (PDGF-BB) (GEM-21®, Osteohealth), bone morphogenetic protein-2 (BMP-2) (INFUSE®, Medtronic), and fibroblast growth factor-2 (FGF-2) (Regroth®, Kaken) are the few clinically approved growths factors with specific

indications for periodontal regeneration and dental implant ridge augmentation. Nevertheless, inconclusive data of clinical efficacy of growth factors in periodontal and bone regeneration has been reported in various studies and high cost of treatment limit the use of growth factor proteins.

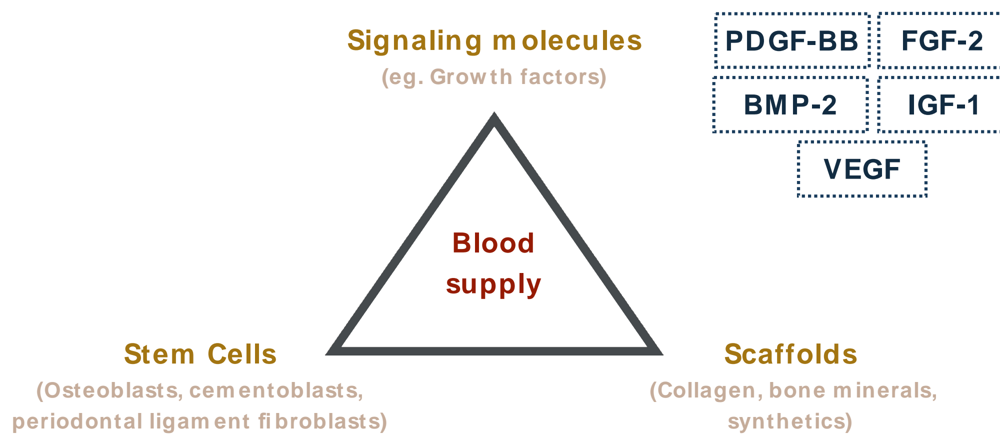

Figure 2. The components of periodontal tissue engineering, including stem cells, scaffolds, signaling molecules and adequate blood supply (modified from Han et al., 2014). PDGF-BB — platelet-derived growth factor, FGF-2 — fibroblast growth factor-2, BMP-2 — bone morphogenetic protein-2, IGF-1 — insulin-like growth factor 1 and VEGF — vascular endothelial growth factor.

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Gene therapy

Instead of delivering growth factor proteins, gene therapy may provide better bioavailability within the damaged tissues, leading to greater tissue regeneration (Franceschi, 2005) This approach involves the delivery of nucleic acids, either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) encoding protein of interest into patient's cells and the cells become the protein factory.

Earlier studies have initiated with plasmid DNA (pDNA) and viral vectors, as messenger RNA (mRNA) was thought to be unstable (Ulmer et al., 1993). The first *in vivo* gene transfer using fibroblast transfected with an adenovirus encoding BMP-7 (Ad-BMP-7) found to promote osteogenesis and cementogenesis in rat alveolar bone defect model (Jin et al., 2004). pDNA encoding BMP-4 complexed with polyethylenimine (PEI) and encapsulated in poly (lactic-co-glycolic acid) scaffold demonstrated significant bone regeneration compared to a scaffold alone in rat cranial defect model (Huang et al., 2005). In contrast, PEI-pDNA encoding PDGF-B polyplexes promoted poor bone regeneration and induced inflammatory cell infiltration (Plonka et al., 2017) The major challenge for using pDNA and viral vectors for periodontitis treatment, a non-life-threatening disease are safety concerns. Inserted DNA could integrate into the host genome, which could pose the risk of mutagenesis. The key discoveries in stability and less innate immunogenicity properties of nucleoside-modified (Karikó et al., 2005) and the efficient lipid encapsulation for mRNA delivery system (Pardi et al., 2015) have greatly contributed to the advancement of mRNA in the field of medicine.

mRNA technology platform

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Application of the *in vitro* transcribed (IVT) mRNA in gene therapy was first described in mice by Wolff and coworkers in 1990. Similar to DNA, injection of IVT mRNA into skeletal muscle led to local production of the protein encoded by the mRNA (Wolff et al., 1990). However, given the fragility of mRNA, it was abandoned as a gene therapy in favor of the more stable pDNA and viral vector.

The major limitations for using mRNA are due to its intrinsic immunogenicity and limited stability (Weissman, 2015). mRNAs bind to innate sensing receptors; TLR3, TLR7, and the RIG-I like receptors, MDA5 and NOD2 (Goubau et al., 2013). This binding activates a pro-inflammatory cytokine response and inhibition of protein translation (Pollard et al., 2013). The immunogenicity of mRNA was

overcome by the use of nucleoside modification (Karikó et al., 2005). The landmark studies in 2005 and 2008 by Kariko and Weissman demonstrated that replacing uridine with pseudouridine into IVT mRNA reduced mRNA-mediated immune activation and inflammation, and simultaneously enhanced protein expression (Karikó et al., 2005; Karikó et al., 2008). In addition, removal of the contaminated dsRNA generated during *in vitro* synthesis by high performance liquid chromatography further reduces immune activation and enhances protein translation (Kariko et al., 2011).

The efficient delivery of mRNA is crucial for successful protein production. mRNA is sensitive to degradation by ribonucleases which are abundant in all living tissues. Encapsulating mRNA with lipids (Mintzer & Simanek, 2009), polymers (Pack et al., 2005), and peptides (Martin & Rice, 2007) enhanced mRNA stability. The most studied RNA delivery system is the use of positively charged or cationic lipids, which has consistently shown good protein expression (Midoux & Pichon, 2015; Mintzer & Simanek, 2009; Pardi et al., 2015). Cationic lipids are used to bind the negatively charged RNA, forming lipoplexes. The lipoplexes could protect mRNA from nucleases, promote endocytosis, and facilitate endosomal escape, enhancing the efficacy of transfection and translation (Guan & Rosenecker, 2017; Kaczmarek et al., 2017; Malone et al., 1989). Due to high efficacy across diverse cell lines and the reproducibility of formulation with cationic lipid liposomes, many commercial products such as Lipofectamine™ and RNAiMAX, have been commonly used for mRNA delivery.

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These key discoveries in stability and less innate immunogenicity properties of nucleoside-modified mRNA (Karikó et al., 2005) and the efficient lipid encapsulation for mRNA delivery system (Pardi et al., 2015) have greatly contributed to the advancement of mRNA in the field of medicine.

After decades of research and development, mRNA has recently emerged as a safe and cost-effective technology platform for developing new class of prophylactic vaccines and therapeutics

(Sahin et al., 2014). mRNA directs the body cells to produce encoding protein of interest *in vivo*. Unlike DNA/viral vectors, mRNA is a non-infectious, non-integrating platform; there is no potential risk of infection or insertional mutagenesis. mRNA encapsulate with lipid-based are taken up via endocytosis. Once inside the cells, mRNA escapes from endosome by ill-defined mechanism and then translated by a ribosome into protein which undergoes post-translational modification to form the mature protein product (Guan & Rosenecker, 2017).

In 2020, the first mRNA product was successfully developed for human use. As a pandemic of coronavirus disease 2019 (COVID-19) was emerged in late 2019 and spread globally leading cause of death over millions people, mRNA vaccine against SARS-CoV-2 spike protein has been rapidly developed (Baden et al., 2021) and approved by the U.S. FDA. This great success will facilitate the development of other mRNA-based vaccines against different infectious diseases including Influenza, Zika and RSV etc. (Jeeva et al., 2021).

mRNA encoding vascular endothelial growth factor in regenerative medicine

Besides a successful mRNA-based vaccine, mRNA-based therapeutics showed promising results in pre-clinical studies. In the field of regenerative medicine, Chien and colleagues were the pioneer researchers investigating mRNA-based therapy for heart tissue regeneration (Chien et al., 2015). In mouse, rat, and pig models of myocardial infarction, intramyocardial injection of modified mRNA encoding VEGF-A165 (VEGF-A mRNA) led to elevated cardiac VEGF-A protein levels and improved heart function and survival, which were associated with improved formation of new blood vessels around the infarct (Carlsson et al., 2018; Zangi et al., 2013). Enhanced differentiation of epicardial progenitor cells toward the endothelial lineage was observed when VEGF-A was delivered with mRNA but not when delivered with a DNA plasmid vector. The same group of researchers

advanced their research into clinical trials. In a human Phase 1 study of type 2 diabetes mellitus (T2DM) patients, Gan and colleagues (2019) found that intradermal injection of VEGF-A mRNA in sucrose citrate buffer showed significantly elevated VEGF-A protein expression and enhanced transient local skin blood flow (Gan et al., 2019). Preliminary data from the phase 2 clinical study in patients undergoing coronary artery bypass grafting suggest positive results that met the primary endpoint of safety and tolerability of VEGF-A mRNA (AstraZeneca, 2021). These seminal studies underscore the therapeutic potential of VEGF-A mRNA for regenerative angiogenesis.

Vascular endothelial growth factor (VEGF)

VEGF is a sub-family of growth factors, the platelet-derived growth factor family of cystine-knot growth factors (Iyer & Acharya, 2011). Of the various growth factors regulating angiogenesis — the formation of new capillaries either by sprouting or splitting (intussusceptive angiogenesis) from the pre-existing vessels. Sprouting angiogenesis occurs when basement membrane of capillary wall is degraded by matrix metalloproteinases (MMPs). Then, endothelial cells migrate, proliferation outside the lumen and form into tubes. For non-sprouting angiogenesis, it is also known as intussusception. It occurs by proliferation of endothelial cells inside a vessel, producing transcapillary pillars that could split pre-existing vessels. Consequently, it leads to form new two isolated blood vessels (Risau 1997).

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VEGF is the most potent agent acting on vascular endothelium (Rufaihah et al., 2017). VEGF also promotes vascular permeability and endothelial cell recruitment, proliferation and differentiation, which play important role in neo-vascularization (Dvorak et al., 1999). In addition, VEGF was shown to be the key factor coupling osteogenesis and angiogenesis since inactivation of VEGF concomitantly subdued blood vessel invasion and bone formation (Clarkin & Gerstenfeld, 2013; Gerber et al., 1999). Therefore, VEGF drives not only angiogenesis, but also osteogenesis, a process of bone formation.

VEGF can be produced by a variety of cells, including endothelial cells, keratinocytes, osteoblasts, macrophages, platelets, renal mesangial cells and tumor cells (Duffy et al., 2013). Hence, this factor could promote cell/tissue growth in physiological (wound healing/regeneration) and pathological (tumor growth) conditions. In periodontal tissue, VEGF was detected in different cell types such as vascular endothelial cells, junctional, sulcular and gingival epithelial cells, macrophages and plasma cells (Booth et al., 1998). Significantly higher VEGF levels were observed in GCF and serum of periodontitis patients than those of healthy periodontal subjects (Padma et al., 2014; Tian et al., 2013). This could be due to the increased inflammatory cytokines, IL-1β and TNF-α, at the sites of active periodontal inflammation, which are known to induce VEGF protein expression (Johnson et al., 1999).

VEGF has been mainly classified into 5 main ligands, including VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF). Each type is subdivided into isoform based on the number of amino acid in the final secreted protein, for example, VEGF 121, VEGF145, VEGF165, VEGF183, VEGF189 and VEGF206 (Ferrara, 2009). The most common is 165-amino-acid isoform of VEGF-A (VEGF-A165). Each VEGF ligand interacts with particular receptors on the endothelial cell called tyrosine kinase, causing them to function differently. VEGF-A could bind to both VEGF receptor 1 (VEGF-R1) and VEGF-R2, so its functions are primarily focused on angiogenesis during homeostasis and disease, whereas VEGF-C and VEGF-D bind to VEGF-R2 and VEGF-R3, implicating in formation of lymphatic capillaries known as lymphagiogenesis (Fig. 3) (Ellis & Hicklin, 2008; Ferrara et al., 2003).

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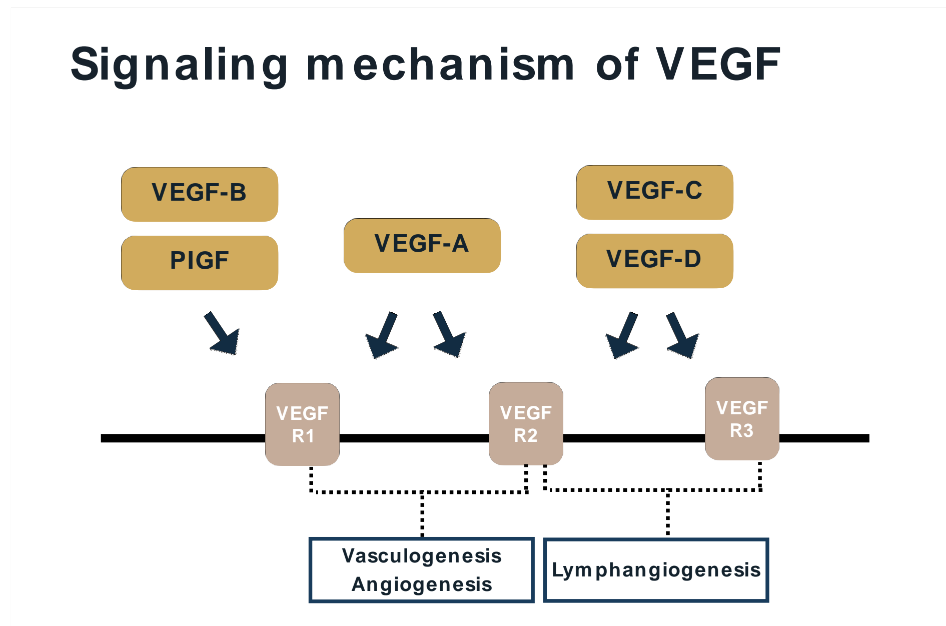

Figure 3. Signaling mechanism of VEGF. VEGF family member (VEGF-A, VEGF-B, VEGF-C, VEGF-C, VEGF-D and PIGF) and their tyrosine kinase (TK) receptors (VEGF-R1, VEGF-R2 and VEGF-R3) have specific binding capabilities, causing them to function differently (modified from Ellis & Hicklin et al., 2008). VEGF — Vascular endothelial growth factor, PIGF — Placental growth factor and VEGF-R — Vascular endothelial growth factor receptor.

Chorioallantoic membrane (CAM) assay

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CAM is a transparent extraembryonic membrane, which is highly vascularized. It is formed by the fusion of chorion and allantois during the embryo development. Its growth starts on day 3 of embryonic development (E3) as a small vesicle and it enlarges very rapidly from E3-E10 of development (Nowak-Sliwinska et al., 2014). The main function of CAM is to serve as not only a breathing organ by providing gas exchange through the pores in the shell, but also an excretory organ by providing a reservoir for waste products such as urea and uric acids. CAM has a role in osteogenesis by drawing calcium from eggshell and regulating the acid-base homeostasis of the embryo (Kundeková et al., 2021). Of importance, the immune system of CAM is not fully developed

until E18, therefore allowing this model to be used for testing various test substances (Kundeková et al., 2021).

In the chick embryo, CAM angiogenesis undergoes three phases of development. In an early phase, sprouting is the major angiogenesis mechanism that occur between E5 to E7. The new blood vessels are spreading into the mesenchymal layer with low density of blood vessels. During the intermediate phase, sprouting is replaced by the intussusceptive angiogenesis between E8 and E12. By E12, the blood vessel system is highly angiogenic with a constant generation of new blood vessels. In the last phase, E13 to E14, the CAM structure is growing and increasing in size with very dense vasculature (Schlatter et al., 1997). Therefore, the appropriate time for testing substances of interest whether being proangiogenic or antiangiogenic by observing the increase or decrease blood vessels on CAM should be between E8 and E10 (Deryugina & Quigley, 2008).

Several CAM assays have been introduced over century ago when rat Jensen sarcoma cells, implanted into the CAM on the day 6 of incubation, were demonstrated to develop large tumors showing signs of tumor-induced angiogenesis (Murphy, 1913). All modifications of the original CAM assay involve grafting of test material onto developing CAM (E8-E-10). The grafting is often performed through a window cut in the eggshell over the CAM. The testing graft material is usually introduced in the form of small disks such as filter papers and collagen soaked in factors of interest. The response of the CAM is normally observed 3 to 5 day post implantation (Deryugina & Quigley, 2008). The response of the angiogenesis could be observed by three ways 1) by counting number of the CAM vessels that appear converging toward the graft in a spoke wheel pattern under stereomicroscope, 2) by analyzing the distribution and density of CAM assay close to the graft, and 3) by analyzing the branching of blood vessels (Ribatti et al., 2006). Due to its simplicity, rapid development and cost-

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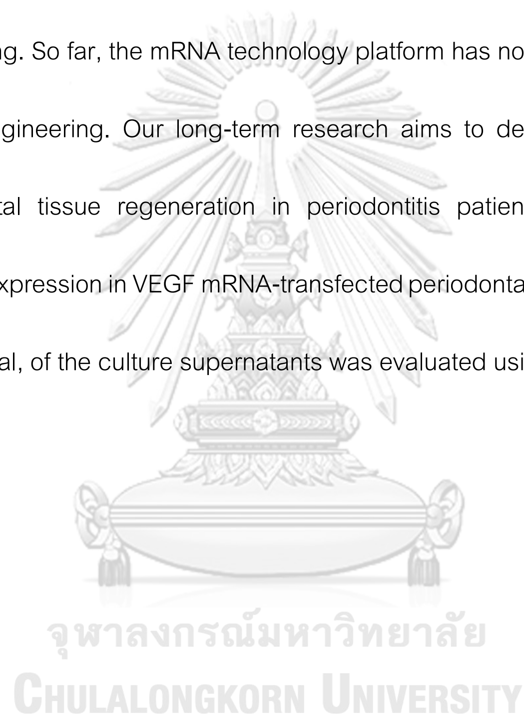
effectiveness, CAM assay has been widely used and recognized as a well-established model for studying angiogenesis, oncology, biology, pharmacy and tissue regeneration.

Targeting stimulation of angiogenesis which is essential for all types of wound healing and tissue regeneration (soft tissue and bone), therapeutic vascular growth has been put forward as a promising strategy for tissue engineering. As mentioned earlier, seminal works utilizing angiogenic growth factor mRNA (VEGF mRNA) demonstrated potential benefits in heart tissue regeneration and diabetic wound healing. So far, the mRNA technology platform has not yet been studied in the field of periodontal tissue engineering. Our long-term research aims to develop mRNA encoding growth factors for periodontal tissue regeneration in periodontitis patients. In this present study, we investigated protein expression in VEGF mRNA-transfected periodontal ligament cells. Then bioactivity — angiogenic potential, of the culture supernatants was evaluated using CAM assay.



CHAPTER III

MATERIALS AND METHODS

Medium and reagents

Minimum Essential Medium with Alpha modification (Alpha MEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM GlutaMax-I, 100 U/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml amphotericin B (Life Technologies) were used throughout the study. Opti-MEM I, Lipofectamine 2000 (L2000) was purchased from Invitrogen. Human recombinant VEGF-A 165 (rhVEGF) was purchased form R&D Systems (Abington, UK). Filter paper was purchased from Whatman, Sigma-Aldrich, Dorset, UK.

Preparation of nucleoside-modified mRNA encoding VEGF

Nucleotide sequence of modified mRNA encoding VEGF-A (VEGF mRNA) designed by Dr. Rangsini Mahanonda as follows.

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VEGF-A gene sequence:

ATGAACTTTCTGCTGTCTTGGGTGCATTGGAGCCTTGCCTTGCTGCTCTACCTCCACCATGCCAAGT
GGTCCCAGGCTGCACCCATGGCAGAAGGAGGAGGGCAGAATCATCACGAAGTGGTGAAGTTCATGGATGTCTA
TCAGCGCAGCTACTGCCATCCAATCGAGACCCTGGTGGACATCTTCCAGGAGTACCCTGATGAGATCGAGTAC
ATCTTCAAGCCATCCTGTGTGCCCCTGATGCGATGCGGGGGCTGCTGCAATGACGAGGGCCTGGAGTGTGTGC
CCACTGAGGAGTCCAACATCACCATGCAGATTATGCGGATCAAACCTCACCAAGGCCAGCACATAGGAGAGAT
GAGCTTCCTACAGCACAACAAATGTGAATGCAGACCAAAGAAAGATAGAGCAAGACAAGAAAATCCCTGTGGG
CCTTGCTCAGAGCGGAGAAAGCATTTGTTTGTACAAGATCCGCAGACGTGTAAATGTTCCTGCAAAAACACAG
ACTCGCGTTGCAAGGCGAGGCAGCTTGAGTTAAACGAACGTACTTGCAGATGTGACAAGCCGAGGCGGTGA

The sequence was sent to TriLink Biotechnologies (USA) for the construction of pseudouridine (Ψ)-modified mRNA encoding VEGF with cap1, DNase and phosphatase treatment, and silica membrane purification, which was packaged as a solution in 1 mM sodium citrate, pH 6.

Isolation and culture of periodontal ligament cells (PDLCs)

The periodontal ligament is a dense connective tissue that connects cementum and alveolar bone to support teeth *in situ*, preserve tissue homoeostasis and provide tissue healing/regeneration (Seo et al., 2004). Successfully isolated multipotent periodontal ligament stem cells from human impacted third molars and these cells could differentiate into periodontal ligaments, alveolar bone, cementum, peripheral nerves, and blood vessels (Huang et al., 2009; Liu et al., 2008; Park et al., 2011). This characteristic of PDLCs is a promising tool for periodontal regeneration and the present study, we used PDLCs as the target cells.

The study protocol was approved by the Ethical Committee (No. 024/2022) and Institutional Biosafety Committee (No. 0621.06/2935) from the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand. All participants were provided written informed consent. PDLCs were harvested from extracted teeth due to orthodontic purposes or therapeutic reasons at the Faculty of Dentistry, Chulalongkorn University. PDLCs were extracted from the tooth by the enzyme-digestion method. The extracted teeth were washed twice with Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco®, Thermo Fisher Scientific, USA) and the PDL tissues were scraped out from the middle third of the root under a sterile condition. Care was exercised to avoid contamination from gingival or periapical granulation tissues. PDL tissues were minced into a fragment of 1-2 mm^2 and immediately placed into a solution of 2 mg/ml collagenase and dispase for 60 min at 37°C for digestion and then filtered through a 70-μm cell strainer. Subsequently, the pass-through was washed twice with the culture medium.

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Then, PDLCs were cultured with the medium (Dulbecco's Modified Eagle Medium (DMEM), Gibco®, Thermo Fisher Scientific, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco®, Thermo Fisher Scientific, USA) at 37°C in a humidified atmosphere of 5% CO_2 in the air. The medium was changed twice weekly. After a confluent monolayer of cells was reached, PDLCs were trypsinized, washed, and then sub-cultured to a new tissue culture flask. The cell from the 3rd to 8th passages of three different donors were used in this study (Iwata et al., 2010; Surisaeng et al., 2020).

In vitro PDLC transfection and production of VEGF protein

In this study, pseudouridine (Ψ)-modified mRNA encoding VEGF (VEGF mRNA) were complexed with Lipofectamine® 2000 (L2000, Invitrogen) (VEGF mRNA-L2000) according to the manufacturer's instructions (Fig. 4). The PDLCs (100,000 cells/well, 24 well tissue culture plate) were transfected with VEGF mRNA-L2000 (1 µg VEGF mRNA, 1.5 µl L2000) or with L2000 only (1.5 µl) (L2000 control) in a final volume of 50 µl. Our previous kinetic study of green fluorescent protein (GFP) mRNA-L2000 showed that peak protein expression occurs at 24 hours (h) transfection in PDLCs (Ratreprasatsuk et al., 2019). Therefore, the culture supernatants in our experiment were collected after 24 h incubation, and VEGF protein production was measured by ELISA kit (Quantikine®, R&D System, Minneapolis, MN, USA) (Fig. 5).

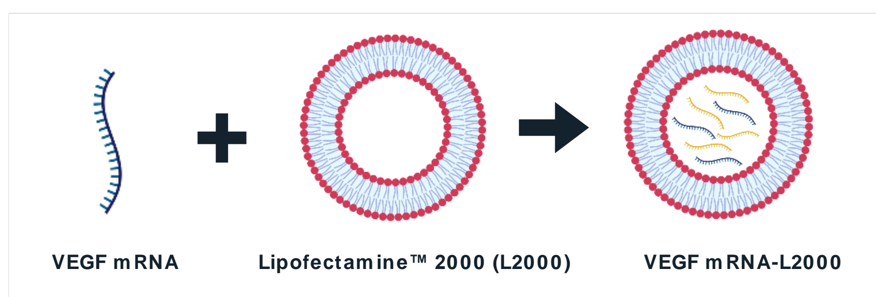

Figure 4. Pseudouridine (Ψ)-modified mRNA encoding VEGF (VEGF mRNA) was complexed with Lipofectamine™ 2000 (L2000)

Cell viability

Besides protein production, cell viability was assessed using Alamar Blue assay after 24 h PDLC transfection. 10% Alamar Blue solution was added to the transfected cells (alamarBlue™, BIO-RAD, CA, USA), then incubate at 37°C in a humidified atmosphere of 5% CO_2 for 4 hours. After incubation, culture supernatants were measured at an absorbance of 570 nm using a microplate reader (Fig. 5) (Epoch™, Biotek™, VT, USA).

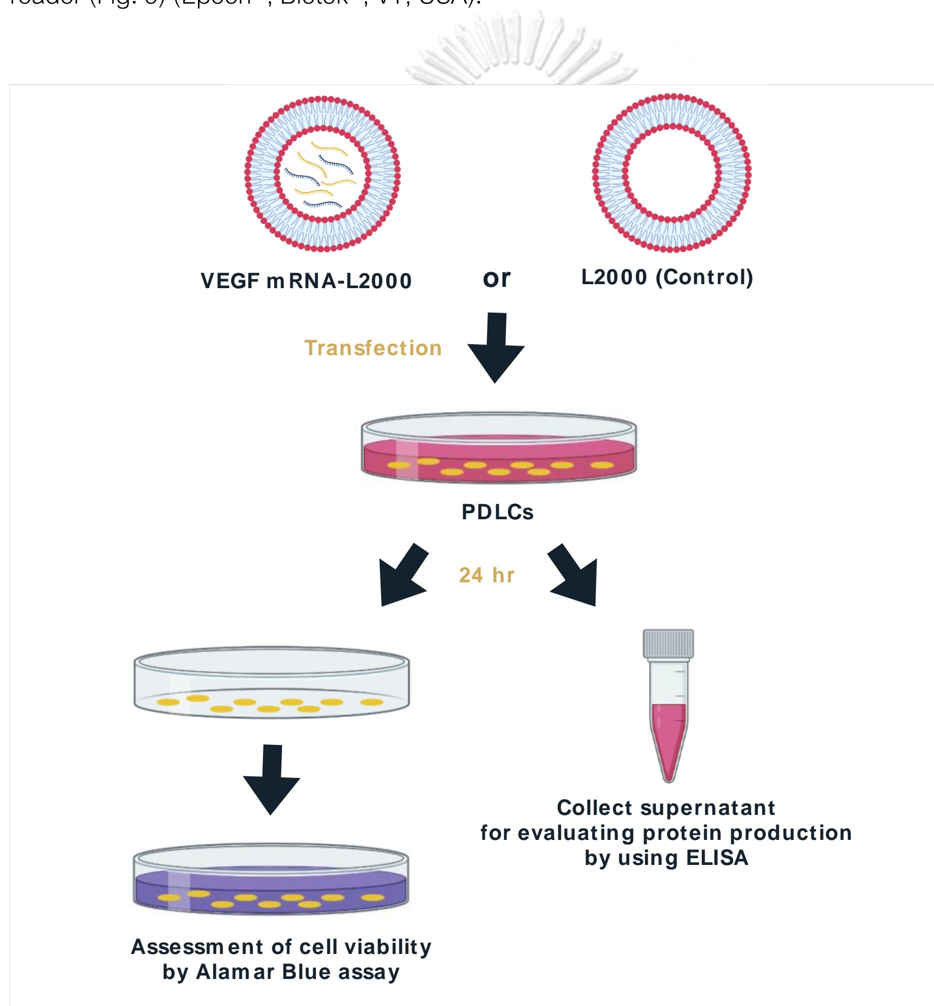

Figure 5. *In vitro* study of PDLC transfection with VEGF mRNA-L2000 and L2000 alone. After 24 hours, culture supernatant was collected for evaluating protein production by ELISA and cell viability was assessed by Alamar Blue assay. L2000 — Lipofectamine™ 2000

CAM experimental model preparation

All animal procedures were conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Science, Chulalongkorn University, Thailand (No. 2031021). In this study, fertilized White Leghorn chicken eggs were obtained from Kasetsart University, Thailand. They were sterilized with 75% ethanol and incubated in an incubator at 37.5° with 60% humidity for the duration of their development

After 3 days of incubation (E3), the eggs were windowed on the eggshell by first aspirating 3 ml of albumen from the blunt end by using a 25G syringe to prevent the shell membrane from adhesion. After removing the albumen, the eggshell was cut like a window (size 10 x 20 mm) by using small dissecting scissors. The window was sealed with transparent tape and returned the egg to the incubator. On day 8 of incubation (E8), filter papers (5 mm diameter, Whatman filter, Grade 41, 220 μm thick with pore size 20-25 μm) with substances of interest (test/control) were individually placed on to the CAM through the window and incubated for three days. In this study, DPBS was used as a negative control. Interestingly, there has been a report from National Center for Biotechnology Information (NCBI) of high-level homology of VEGF-A between human and chicken. The eggs were randomly divided into 4 groups (7 eggs per group, total eggs = 28 eggs) (Ribatti et al., 2006; Rumney et al., 2019) as follows:

<u>Group 1</u> (negative control): Whatman with DPBS (10 μl)

<u>Group 2</u> (rhVEGF) : Whatman with rhVEGF (500 ng/10 μl)

<u>Group 3</u> (L2000 control) : Whatman with supernatant from L2000 control transfected PDLCs (at a predetermined dilution of 1:40) (Supplementary Appendix; Table 3, Fig. 1)

<u>Group 4</u> (mRNA) : Whatman with supernatant from VEGF mRNA-L2000 transfected PDLCs (at a predetermined dilution of 1:40) (Fig. 6, Supplementary Appendix; Table 3, Fig. 1)

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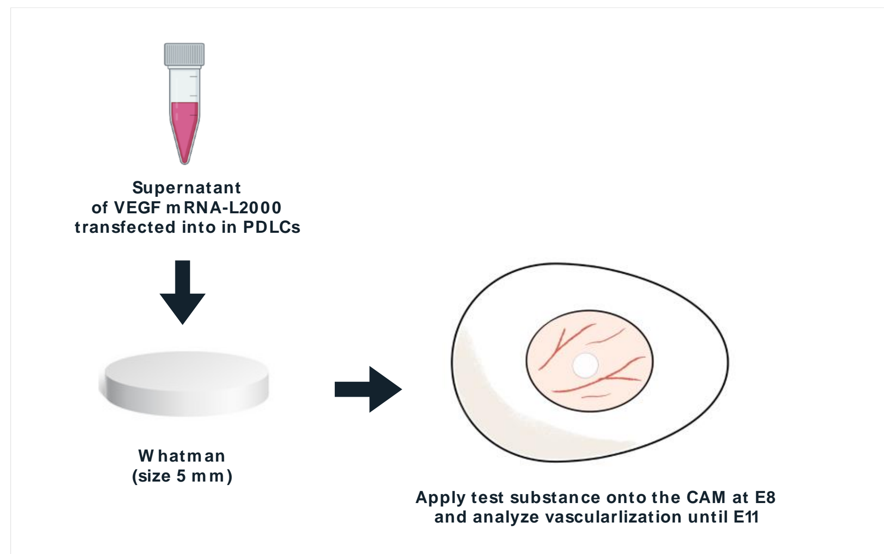

Figure 6. CAM assay testing translated protein from VEGF mRNA-L2000 transfected PDLCs. Implantation of Whatman filter paper with VEGF mRNA-L2000 was placed onto the CAM at E8 and the increased number of blood vessels were counted on E8 and E11.

After placement of the filter papers with substances of interest, the window was again covered with transparent tape and returned to the incubator. The evaluation was carried out by photographing with stereomicroscopic on E8 and E11. On day 11 of incubation (E11), all the eggs were euthanized (Fig. 7) (Ribatti et al., 2006).

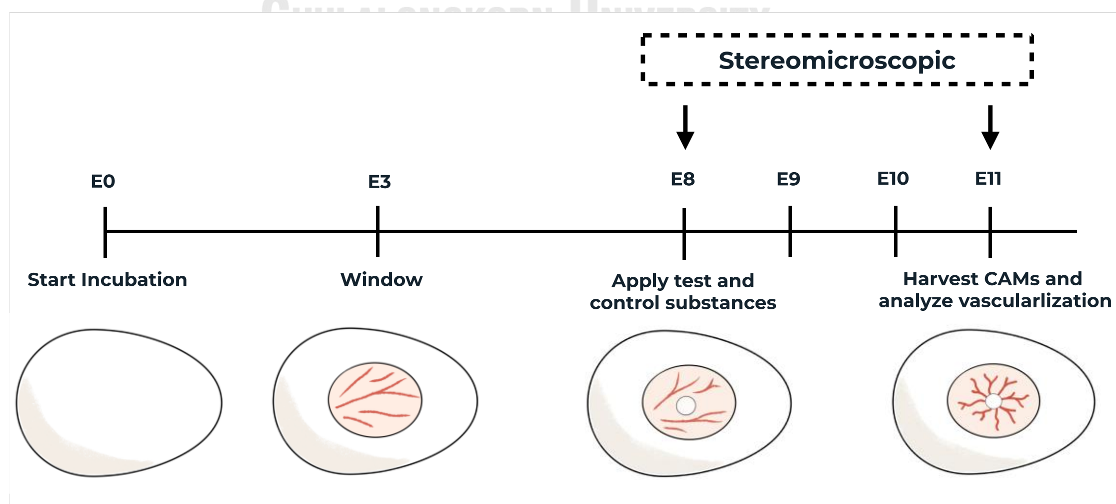

Figure 7. Diagram showing the timeline of the experiment in CAM assay.

Stereomicroscopic evaluation

After the implantation of the material, the angiogenic response can be evaluated by counting the total number of the microvessels (3-10 μm) that convergence toward the grafts (filter plus substance of interest) under a stereomicroscope (Olympus Stereomicroscope (SZ61), Rozzano, Italy) at 10x magnification on E8 and E11 (Ribatti et al., 2006). Angiogenic response was characterized by an increased number of blood vessels. All the images were generated in Otsu grey threshold using ImageJ software (National Institutes Health, Bethesda, MD, USA). The counting was done by three independent observers in a blind manner (Ribatti et al., 2006). Intraclass correlation coefficient (ICC) from all group range 0.95-0.98 which indicated excellent reliability (Koo & Li, 2016).

Statistical analysis

The statistical software SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Normal distribution of data was evaluated by Shapiro-Wilk normality test. Homogeneity of variance was accessed by Levene's test. Differences among groups were evaluated by one-way ANOVA followed by Games-Howell post-hoc analysis. For all statistical analysis, p-value less than 0.05 were considered statistically significant.

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CHAPTER IV

RESULTS

VEGF protein production in pseudouridine (Ψ)-modified mRNA encoding VEGF complexed with Lipofectamine 2000 transfected PDL cells

PDLCs were transfected with Ψ-modified mRNA encoding VEGF complexed with Lipofectamine 2000, while Lipofectamine alone was used as controls. After collected supernatants from the cultures at 24 h, quantification of secreted VEGF protein using ELISA revealed the mean concentration of VEGF in mRNA group was 25,105 ± 1,326.84 pg/ml and 545.93 ± 25.69 pg/ml in L2000 control group (Fig. 8, Supplementary Appendix; Table 1). Levels of VEGF protein in mRNA group was significantly higher than L2000 control group ($p < 0.001$, $n = 3$).

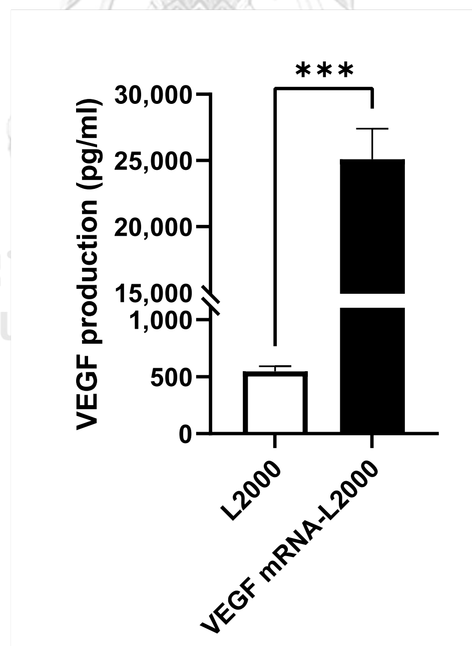

Figure 8. *In vitro* production of VEGF protein from PDLCs in VEGF mRNA-L2000 group vs L2000 control group. Data are shown as mean ± SE ($n = 3$). *** $p < 0.001$ indicate a significant difference between the mRNA and the control groups.

Cell viability following the VEGF mRNA transfection complexed with L2000

The cell viability after VEGF mRNA transfection in PDLCs was assessed after 24 h incubation by Alamar Blue assay. It was found the cell viability greater than 85 percent was observed amongst the mRNA and control groups (Fig. 9, Supplementary Appendix; Table 2).

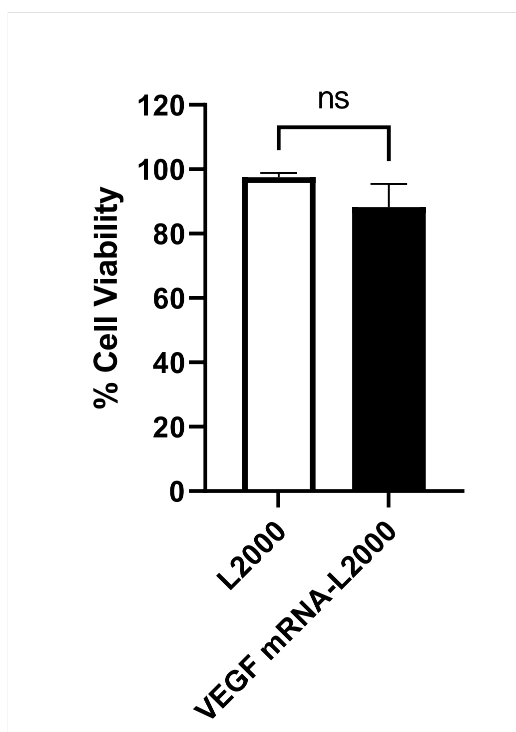

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Figure 9. Cell viability after transfection with VEGF mRNA-L2000. Data are shown as mean ± SE of cell viability of transfected PDLCs comparing between mRNA group and L2000 control groups after 24 h incubation (*n* = 3). NS = not significant (*p* = 0.094).

Effect of VEGF mRNA-L2000 transfected PDLCs upon angiogenesis in the CAM assay

Following measuring the *in vitro* protein expression, the biological function of translated protein in the culture supernatants of the mRNA and L2000 control groups was tested *in vivo* for its ability to induce blood vessel formation using CAM assay.

It should be noted that the cell culture medium in this experiment contained 10% fetal bovine serum (FBS). The FBS enriches with nutrient such as growth factors, hormones, vitamins, and other nutrients that can support the growth, proliferation and viability of cells in culture, therefore possibly affecting the capacity of VEGF mRNA to induce blood vessel formation. Hence, we needed to determine the proper concentrations of culture supernatants of VEGF mRNA-L2000 and L2000 by testing different dilutions, 1:2; 1:10; 1:20; 1:40; 1:80 of these supernatants in CAM assay. The results of mean number of increased blood vessels were as follows: 1) 1:2 dilution was 39.94 ± 4.93 in L2000 group vs 29.47 ± 3.42 in mRNA group, 2) 1:10 dilution was 41.00 ± 4.44 in L2000 group vs 40.00 ± 1.75 in mRNA group, 3). 1:20 dilution was 40.40 ± 6.77 in L2000 group vs 38.14 ± 5.29 in mRNA group, 4). 1:40 dilution was 31.73 ± 1.65 in L2000 group vs 50.53 ± 1.18 in mRNA group, 5). 1:80 dilution was 36.93 ± 5.52 in L2000 group vs 36.07 ± 4.36 in mRNA group. The significant differences of the mean increased blood vessels between mRNA group and L2000 control group were found only in 1:40 dilution ($p < 0.0001$) (Supplementary Appendix; Table 3, Fig. 1). Therefore, 1:40 dilution was used to compare the supernatant groups with those DPBS and rhVEGF.

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Figure 10 shows a representative of stereomicroscope image of CAM at E8 and E11 after placing filter papers with DPBS, rhVEGF, supernatant from L2000 control and supernatant from VEGF mRNA-L2000 (1:40 dilution). On E8, a small number of blood vessels in the CAM were detected in all groups (Fig. 10a, 10b). The mean number of blood vessels were in 20.81 ± 2.14 in DPBS group, 23.35 ± 1.67 in rhVEGF group, 24.48 ± 2.26 in L2000 control group, 22.62 ± 1.42 in mRNA group (Supplementary Appendix; Table 4).

On E11, more abundant microvascular networks as a spoked-wheel pattern were observed in mRNA group and rhVEGF group than in L2000 group and DPBS group (Fig. 10a, 10b). The mean

number of increased blood vessels were 31.38 ± 3.00 in DPBS group, 42.86 ± 5.62 in rhVEGF group, 32.67 ± 1.96 in L2000 control group, 51.38 ± 2.23 in mRNA group. mRNA group significantly increased the number of blood vessels as compared to DPBS groups and L2000 control group (*p <0.001*), but not to rhVEFG group (Fig. 10c, Supplementary Appendix; Table 8).

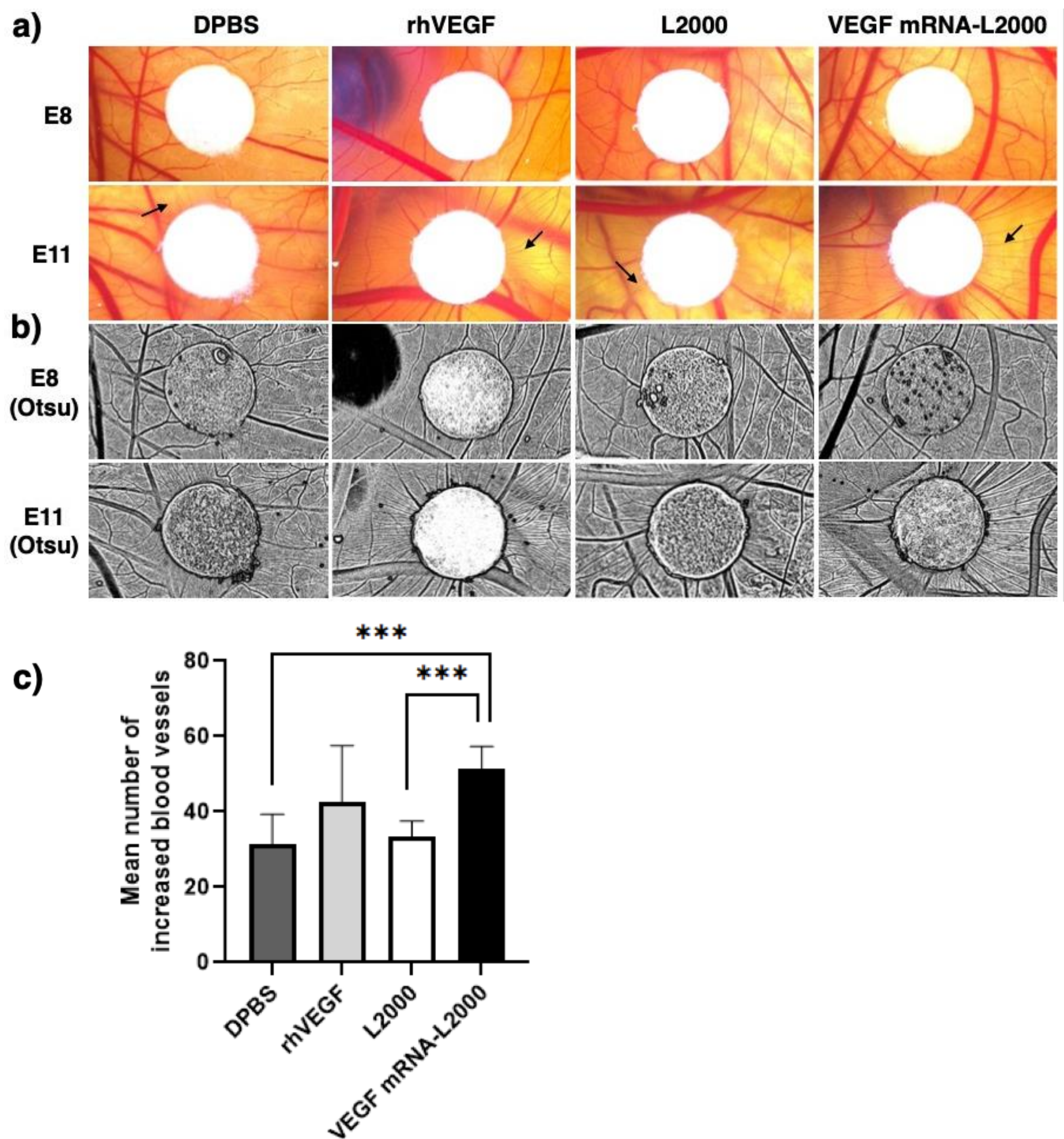

Figure 10. Chick embryo chorioallantoic membrane (CAM) assay. (a) A representative of stereomicroscope image of CAM at E8 and E11 after placing Whatman filters with DPBS, rhVEGF,

supernatant from L2000 control and supernatant from VEGF mRNA-L2000. On E11, more abundant microvascular networks as a spoked-wheel pattern (arrows) were observed in mRNA group (Original magnification, 10x). **(b)** Image analysis of CAM at E8 and E11 of individual control/test was generated by using Otsu technique. **(c)** The mean number of increased blood vessels from E8 to E11 in DPBS, rhVEGF, L2000 control and mRNA groups. mRNA group significantly increased the number of blood vessels as compared to the controls (DPBS and L2000). Data are shown as mean ± SE (n = 7). *** $p < 0.001$ indicate a significant difference between the mRNA and the control groups (DPBS and L2000).

CHAPTER V

DISCUSSION AND CONCLUSION

This study is the first study to explore the potential use of mRNA encoding VEGF in periodontal tissue regeneration. *In vitro* transfection of PDLCs with pseudouridine-modified mRNA in L2000 demonstrated high production of VEGF protein. This translated protein functioned, showing the ability to induce significant blood vessel formation using an *in vivo* CAM assay.

In this study, PDLCs were used due to their stemness potential that can differentiate into a variety of cells including osteoblasts and cementoblasts, important cells for periodontal regeneration (Seo et al., 2004). Our transfection results support previous data in our laboratory (the Excellent Center for Periodontal Disease and Dental Implant) that used other growth factor mRNAs. With similar culture conditions (100,000 PDLCs/well in 24-well plate), all reported high protein expression after 24 h PDLC transfection with corresponding growth factor modified mRNA complexed with transfecting agent L2000. The mean VEGF protein production in our experiment was approximately 25,105 ± 1,326.84 pg/ml with 85% cell viability, while Surisaeng et al (2020) showed PDGF-BB protein expression of 26,815 ± 7,343.31 pg/ml and with 90% cell viability in PDGF-BB mRNA- transfected PDLCs and Kulthanaamondhita et al (2020) revealed BMP-2 protein expression of 12,285 ± 6,321.95 pg/ml with 90% cell viability in BMP-2 mRNA-transfected PDLCs (Kulthanaamondhita et al., 2020; Surisaeng et al., 2020). The mRNA technology is recognized as a simple plug-and-play technology that allows us to change the coding sequence of different genes of interest. It would be a simple but powerful tool to generate different growth factors to treat periodontal tissue regeneration.

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In vivo study, CAM assay was conducted to evaluate the biological activity, angiogenesis, of translated VEGF production in PDLC culture supernatants. The VEGF mRNA group shows a significant increase in the blood vessel network, with a radial arrangement of blood vessels directed toward the graft like a spoked-wheel pattern as compared to the L2000 control and DPBS groups. Our results agreed with a previous study, where they used VEGF mRNA transfected in human osteoblast-like MG-63 cells seeded on polycaprolactone scaffold and placed on CAM (Rumney et al., 2019). They found the combined mRNA technology with cell therapy and scaffold could lead to a markedly increased angiogenesis. Our data of rhVEGF group also showed a trend of dense blood vessel network, but the mean number of increased blood vessels were not markedly differences from both control groups. The rhVEGF concentration of 500 ng used in our experiment was selected from previous studies, ranging from 0.6 – 1,000 ng (Rumney et al., 2019; Singh et al., 2012; Zhang et al., 2012). If the higher rhVEGF concentration were used, the positive effect may have been more prominent.

Interestingly, the concentration of translated VEGF protein in culture supernatant of the mRNA group was 0.148 ng, which was markedly lower than that of the rhVEGF group (500 ng). The possible explanation would be that in recombinant protein technology, the proteins are usually produced from different species including insects and *E. coli* (Gasser et al., 2008). As a result, there are abundance of misfold polypeptides, which cause stress in host cells. In contrast, mRNA uses host cells as a factory to endogenously produce properly folded protein (Zhang et al., 2019), thus avoiding unwanted stress to host cells and the translated protein is biological active.

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However, we experienced some limitations during CAM experiment. A contamination of CAM from the eggshell dust during the make of eggshell window could occur. This may cause inflammation and unwanted CAM reaction leading to result misinterpretation. We avoided this incident by cutting

eggshell carefully with sharp surgical scissor, instead of drilling. However, if the contamination accidentally occurs, the data should be excluded. In another scenario, the chick embryo grew larger or moved to filter paper area which could interfere the counting process, or in other words impossible to count. Again, we excluded those subjects. Despite these limitations, we found the CAM assay is valuable for assessing angiogenesis and provides a good experimental system for testing the potential use of mRNA in tissue regeneration.

VEGF is an important signaling protein involved in promoting the growth of new blood vessel formation that provides oxygen and nutrients to survive. Moreover, VEGF was shown to be the key factor coupling osteogenesis and angiogenesis since the inactivation of VEGF concomitantly subdued blood vessel invasion and bone formation (Clarkin et al., 2013; Gerber et al., 1999). The potential of mRNA therapeutics in stimulating VEGF protein production to provide reparative and regenerative effect has been demonstrated in the area of soft tissue and bone. The most advanced and exciting area is VEGF mRNA application for heart tissue regeneration, which is now ongoing in Phase 3 clinical trial in cardiovascular patients (Collén et al., 2022). Periodontium is a complex functional tissue consisting of soft tissues and bone. Even tough, angiogenesis plays an important role in soft and hard tissue regeneration, the success in periodontal tissue regeneration may require mRNA encoding VEGF with additional growth factor such as a strong osteogenic factor – BMP-2 mRNA. A recent study by Geng et al. (2021) demonstrated VEGF mRNA in combination with BMP-2 mRNA, which was delivered with cell therapy and the use of scaffold, could enhance significant bone formation in a preclinical model, a rat calvarial bone defect (Geng et al., 2021).

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The next step in the development of this mRNA therapeutic platform is to further evaluate the ability of VEGF mRNA to promote soft and bone tissue regeneration in larger animal models and combination of growth factors could also be assessed.

In conclusion, we demonstrated that modified mRNA encoding VEGF can promote VEGF production after transfected in PDLCs with negligible effect on cell viability. This translated protein had angiogenic property to promote the formation of blood vessels in the CAM. Thus, this mRNA could be beneficial in the treatment of periodontal regeneration.

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APPENDIX

Supplementary Table 1. *In vitro* VEGF protein production from PDLCs in VEGF mRNA-L2000 group, L2000 control group and medium control.

VEGF-A production (pg/ml)	Control	L2000	VEGF mRNA L2000
PDLCs1	449.60	592.90	23,005.00
PDLCs2	288.30	540.50	24,750.00
PDLCs3	501.40	504.40	27,560.00
Mean ± SE	413.10 ± 64.17	545.93 ± 25.69	25,105.00 ± 1,326.84*

Data are shown as mean ± SE ($n = 3$).

*$p < 0.001$ indicate a significant difference between the mRNA and the control groups.

Supplementary Table 2. Cell viability after transfection with VEGF mRNA-L2000, L2000 control group and medium control after 24 h incubation

Toxicity (%)	Control	L2000	VEGF mRNA L2000
PDLCs1	100.00	96.18	80.27
PDLCs2	100.00	98.67	90.10
PDLCs3	100.00	97.89	94.35
Mean ± SE	100.00 ± 0.00	97.58 ± 0.74	88.24 ± 4.17

Data are shown as mean ± SE ($n = 3$).

NS = not significant ($p = 0.094$).

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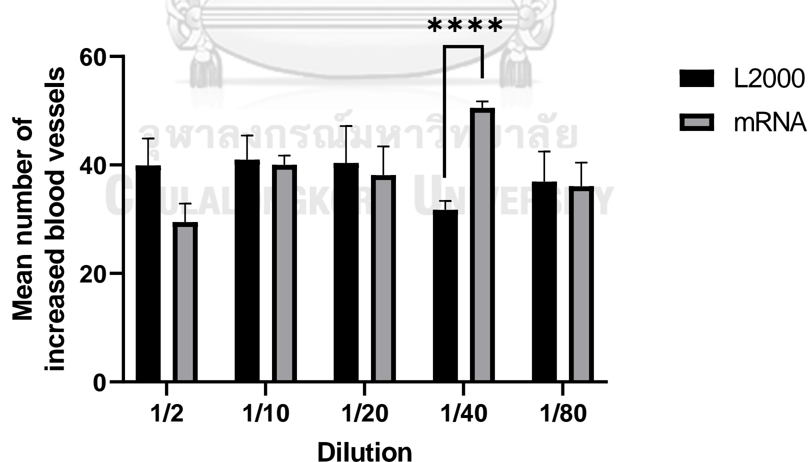
Supplementary Table 3. The mean number of increased blood vessels from E8 to E11 of supernatant of VEGF-mRNA and L2000 (control) in various dilutions.

Groups (dilutions)	L2000 (1/2)	mRNA (1/2)	L2000 (1/10)	mRNA (1/10)	L2000 (1/20)	mRNA (1/20)	L2000 (1/40)	mRNA (1/40)	L2000 (1/80)	mRNA (1/80)
1	41.00	38.00	43.66	40.00	25.00	57.33	33.00	50.00	53.67	39.00
2	24.67	24.00	52.33	37.67	60.00	35.67	29.00	53.33	42.33	51.67
3	47.33	35.33	41.30	41.33	49.67	27.67	31.33	50.00	23.67	32.00
4	52.70	19.67	42.70	35.33	26.00	40.33	37.33	52.67	26.00	27.00
5	34.00	30.33	25.00	45.67	41.33	29.70	28.00	46.67	39.00	30.67
Mean	39.94	29.47	41.00	40.00	40.40	38.14	31.73	50.53 *	36.93	36.07
SE	4.93	3.42	4.44	1.75	6.77	5.29	1.65	1.18	5.52	4.36

Data are shown as mean ± SE ($n = 5$).

*$p < 0.001$ indicate a significant difference between the mRNA group and L2000 (control) group in 1:40 dilution only.

Supplementary Figure 1. The mean number of increased blood vessels from E8 to E11 of supernatant of VEGF-mRNA and L2000 (control) in various dilutions.



Data are shown as mean ± SE ($n = 5$).

****$p < 0.0001$ indicate a significant difference between the mRNA group and L2000 (control) group in 1:40 dilution only.

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Supplementary Table 4. The mean number of blood vessels of DPBS groups, rhVEGF group, L2000 group, mRNA group on E8.

	DPBS	rhVEGF	L2000	VEGF mRNA L2000
1	15.00	23.67	18.67	21.67
2	12.00	24.33	19.67	15.67
3	27.00	25.67	34.33	22.67
4	25.66	22.00	28.67	22.00
5	22.00	26.67	28.33	27.00
6	19.33	27.00	21.67	22.67
7	24.67	14.33	20.00	26.67
Mean ± SE	20.81 ± 2.14	23.35 ± 1.67	24.48 ± 2.26	22.62 ± 1.42

Data are shown as mean ± SE (n = 7).

Supplementary Table 5. The mean number of blood vessels of DPBS groups, rhVEGF group, L2000 group, mRNA group on E9.

	DPBS	rhVEGF	L2000	VEGF mRNA L2000
1	19.00	34.67	27.67	32.00
2	15.33	30.33	28.33	29.67
3	34.00	31.67	34.67	33.67
4	35.00	37.33	28.67	30.67
5	25.67	28.67	28.33	33.33
6	20.00	30.00	29.33	34.33
7	22.00	18.33	26.00	38.67
Mean ± SE	24.33 ± 2.86	30.14 ± 2.27	29.00 ± 1.02	32.89 ± 0.90

Data are shown as mean ± SE (n = 7).

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Supplementary Table 6. The mean number of blood vessels of DPBS groups, rhVEGF group, L2000 group, mRNA group on E10.

	DPBS	rhVEGF	L2000	VEGF mRNA L2000
1	24.67	45.00	41.33	63.00
2	21.00	39.50	39.33	35.00
3	45.67	49.67	61.00	45.00
4	44.33	65.67	48.00	48.67
5	48.00	55.33	60.33	70.33
6	48.33	53.00	50.67	55.00
7	23.00	59.00	36.67	53.67
Mean ± SE	36.43 ± 4.83	52.45 ± 3.30	48.19 ± 3.70	52.95 ± 4.40

Data are shown as mean ± SE (*n* = 7).

Supplementary Table 7. The mean number of blood vessels of DPBS groups, rhVEGF group, L2000 group, mRNA group on E11.

	DPBS	rhVEGF	L2000	VEGF mRNA L2000
1	39.33	62.67	52.00	71.67
2	38.33	71.00	44.00	69.00
3	51.00	57.33	65.67	72.67
4	65.67	91.00	66.00	74.67
5	59.00	48.00	56.33	73.67
6	61.33	74.67	60.33	85.33
7	50.67	59.00	56.00	71.00
Mean ± SE	52.19 ± 4.00	66.24 ± 5.31	57.19 ± 2.94	74.00 ± 2.01

Data are shown as mean ± SE (*n* = 7).

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Supplementary Table 8. The mean number of increased blood vessels from E8 to E11 in DPBS control, L2000 control, and mRNA groups.

	DPBS	rhVEGF	L2000	VEGF mRNA L2000
1	24.33	39.00	33.00	50.00
2	26.33	46.67	24.33	53.33
3	24.00	31.67	31.33	50.00
4	40.00	69.00	37.33	52.67
5	37.00	21.33	28.00	46.67
6	42.00	47.67	38.67	62.67
7	26.00	44.70	36.00	44.33
Mean ± SE	31.38 ± 3.00	42.86 ± 5.26	32.67 ± 1.96	51.38 ± 2.23*

Data are shown as mean ± SE ($n = 7$).

*$p < 0.001$ indicate a significant difference between the mRNA and the control groups (DPBS, L2000).

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VITA

NAME	มานา นราธิปกร
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PLACE OF BIRTH	กรุงเทพมหานคร
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